Estrogen receptor β and 17β-hydroxysteroid dehydrogenase type 6, a growth regulatory pathway that is lost in prostate cancer

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Estrogen receptor β (ERβ) is activated in the prostate by 5α-androstane-3β,17β-diol (3β-Adiol) where it exerts antiproliferative activity. The proliferative action of the androgen receptor is activated by 5α-dihydrotestosterone (DHT). Thus, prostate growth is governed by the balance between androgen receptor and ERβ activation. 3β-Adiol is a high-affinity ligand and agonist of ERβ and is derived from DHT by 3-keto reductase/3β-hydroxysteroid dehydrogenase enzymes. Here, we demonstrate that, when it is expressed in living cells containing an estrogen response element-luciferase reporter, 17β-hydroxysteroid dehydrogenase type 6 (17βHSD6) converts the androgen DHT to the estrogen 3β-Adiol, and this leads to activation of the ERβ reporter. This conversion of DHT occurs at concentrations that are in the physiological range of this hormone in the prostate. Immunohistochemical analysis revealed that 17βHSD6 is expressed in ERβ-positive epithelial cells of the human prostate and that, in prostate cancers of Gleason grade higher than 3, both ERβ and 17βHSD6 are undetectable. Both proteins were present in benign prostatic hyperplasia samples. These observations reveal that formation of 3β-Adiol via 17βHSD6 from DHT is an important growth regulatory pathway that is lost in prostate cancer.

HSD17B6 | short-chain dehydrogenase | steroid metabolism | prereceptor regulation | intracrinology

Estrogen receptor β (ERβ) is a member of the nuclear receptor superfamily of transcription factors (1). ERβ is activated by its endogenous ligands estradiol-17β (E2) and 5α-androstane-3β,17β-diol (3β-Adiol). Activated ERβ is known to antagonize the proliferative actions of ERα (2–4). It has been shown that, in the prostate, 3β-Adiol is the physiological ligand of ERβ (2). This notion is supported by the fact that the intraprostatic 3β-Adiol level (10 nM) is 100-fold higher than that of E2 (0.1 nM) (5). 3β-Adiol is a metabolite of the androgen receptor (AR) agonist 5α-dihydrotestosterone (DHT).

In vitro experiments using membrane preparations or purified enzymes have demonstrated that 3β-Adiol can be formed from DHT in two ways: (*i*) directly by 3-keto reduction of DHT to 3β-Adiol or (*ii*) by a two-enzyme process that entails a 3-keto reduction to 3α-Adiol followed by 3α- to 3β-hydroxysteroid epimerization.

The most likely candidate enzyme for direct reduction is AKR1C1, a member of the aldo-keto reductase (AKR) family (6, 7). This cytosolic enzyme predominantly catalyzes the conversion of DHT to 3β-Adiol (8). A candidate for the first step in the two-enzyme pathway is AKR1C2, which converts DHT to 3α-Adiol (9). The mRNAs for both enzymes have been quantified in the human prostate by quantitative PCR (10); however, their cell type-specific protein expression in the prostate has not been reported.

The epimerase reaction has been shown to be catalyzed by 17β-hydroxysteroid dehydrogenase type 6 (17βHSD6) (11, 12), also termed retinol dehydrogenase-like 3α-hydroxysteroid dehydrogenase (13, 14). 17βHSD6 is a microsomal enzyme that belongs to the short-chain dehydrogenase superfamily whose mRNA is expressed in the human and rat prostate (11); however, the protein's cell type-specific expression has not been described. We show here that 17βHSD6 has the capacity to directly convert physiological concentrations of DHT to 3β-Adiol with a concomitant activation of ERβ. The biological significance of 17βHSD6 acting as a 3β-Adiol synthase is supported by the fact that the enzyme colocalizes with ERβ protein in epithelial cells of the human prostate.

Results

Effect of 17βHSD6 on ERβ Transactivation by 5α-Reduced Steroids. Because DHT, 3β-Adiol, and 3α-Adiol are present at ∼20, 10, and 2 nM concentrations, respectively, in the human prostate (5), coupled with the fact that 3β-Adiol is a potent ligand of ERβ in this tissue, we asked the question whether 17βHSD6 has the capacity to form sufficient amounts of 3β-Adiol from DHT and 3α-Adiol to activate ERβ in living cells. A transactivation assay in which human ERβ was expressed in human embryonic kidney 293 cells (HEK-293 cells) harboring an ERβ-responsive estrogen response element (ERE) containing promoter/reporter plasmid was used. Fig. 1 shows that coexpression of 17βHSD6 resulted in ERβ activation when DHT (20 nM) was added to the cells; 3α-Adiol also caused a robust activation of ERβ in a 17βHSD6-dependent fashion. ERβ activation by estradiol and 3β-Adiol was not affected by coexpressing 17βHSD6. Fig. 2 shows dose–response curves for ERβ activation by DHT and 3α-Adiol in the presence of recombinant 17βHSD6; the EC_{50} values were determined to be ∼20–30 nM for both steroids. Cells transfected with empty vector also demonstrated significant ERβ activation; the presence of an endogenous epimerase activity in HEK-293 cells has previously been reported (14).

Steroid Metabolism by 17βHSD6. To confirm that 17βHSD6 produces 3β-Adiol from DHT, we expressed 17βHSD6 in HEK-293 cells and performed a time-course experiment. Forty-eight hours after transfection, [3H]-DHT was added to the cell medium at a physiological concentration of 20 nM. Samples were collected after 0, 1, 2, 4, and 8 h of incubation. Thereafter steroids were extracted from the medium and separated by TLC on silica gel plates followed by quantitative analysis. Fig. 3 shows that ∼13% of the DHT was converted to 3β-Adiol after 4 h of incubation. As previously reported (11), 17-keto metabolites accumulate over time in 17βHSD6-expressing cells. Mock-transfected cells demonstrated significantly lower DHT metabolism at the 4 h time point. At the end of 8 h, ∼15–20% of DHT was remaining in the medium from cells transfected with 17βHSD6 compared with ∼65–75% in the medium from cells transfected with pCMV vector.

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The authors declare no conflict of interest.

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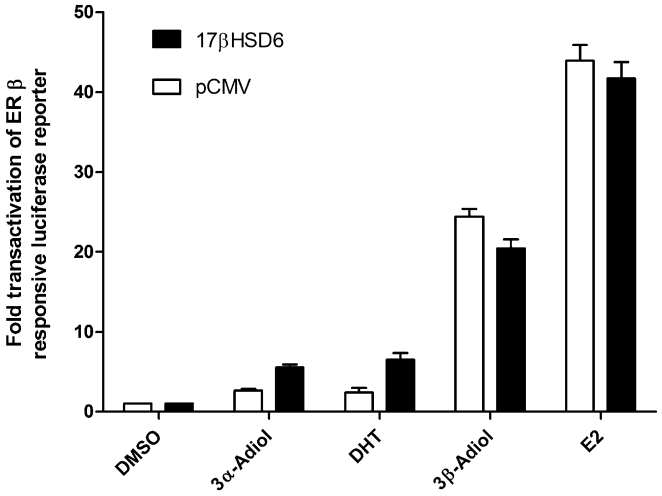

Fig. 1. Coexpression of 17βHSD6 increases ERβ transactivation by DHT and 3α-Adiol. Expression plasmids of ERβ, β-galactosidase, and 17βHSD6 or empty vector were transfected together with an ERE–luciferase reporter plasmid into HEK-293 cells. Luciferase and β-galactosidase values were measured after incubation with 20 nM steroids for 16 h. Fold activation is obtained by calculating luciferase–β-galactosidase and normalizing it with the ratio for the DMSO control. Values are expressed as the mean ± SEM of triplicate wells. Data are representative of three independent experiments.

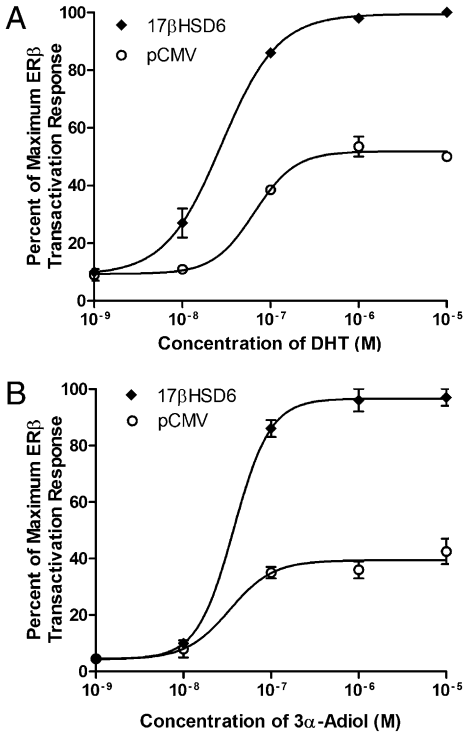

Fig. 2. Dose–response curves for ERβ transactivation with DHT (*A*) and 3α-Adiol (*B*) as substrates in the presence of 17βHSD6 and empty vector. DHT activated ERβ with $EC_{50} \sim 20$ nM with a maximum fold activation of 12.5. 3α-Adiol activated ERβ with $EC_{50} \sim 30$ nM with a maximum fold activation of 21. Values are expressed as the mean ± SEM of triplicate wells. Data are representative of three independent experiments.

Immunohistochemical Analysis of 17βHSD6 in Human Prostate. In an effort to assess the relevance of the enzyme in the human prostate, we determined expression of 17βHSD6 and ERβ in human prostate cancers of different Gleason grades (15) and in benign prostatic hyperplasia (BPH) (16). The immunostaining results from the antibody raised against the N-terminal amino acids 1–50 (Fig. 4 *A* and *B*) were confirmed with the antibody raised against the full-length protein. Immunohistochemical analyses of human prostate sections demonstrated perinuclear staining of 17βHSD6 protein in the epithelial cells of benign prostatic hyperplasia, especially in the basal epithelial cells (Fig. 4 *A* and *B*). Slides incubated without primary antibody demonstrated no staining. As expected (17), sections stained with an antibody against ERβ showed nuclear staining for ERβ protein in the epithelial cells (Fig. 4 *C* and *D*).

We found that, compared with BPH prostate, expression of ERβ decreased in prostate cancers of Gleason grade 3 (Fig. 5*A*) and was undetectable in grades 4 (Fig. 5 *B* and *C*) and 5 (Fig. 5*D*). Expression of 17βHSD6 paralleled that of ERβ in these prostate cancers with a reduction of the immunoreactivity in Gleason grade 3 (Fig. 6 *A* and *B*) and loss of immunoreactivity in grades 4 (Fig. 6 *C* and *D*) and 5 (Fig. 6 *E* and *F*).

Discussion

3β-Adiol is a high-affinity agonist for ERβ on the basis of in vitro ligand-binding and cell-based transactivation assays. In this study, we demonstrate that 17βHSD6 is an enzyme that in living cells converts the androgen DHT to the estrogen 3β-Adiol with a concomitant activation of ERβ and that 17βHSD6 is expressed in ERβ-positive epithelial cells of the human prostate. These observations suggest that, in the formation of 3β-Adiol from DHT, 17βHSD6 is a key enzyme and that 17βHSD6 expression in ERβ-expressing cells is a prereceptor mechanism for ERβ action (Fig. 7).

Prereceptor regulation of hormone action is an important concept in endocrinology. To understand the mechanism by which the intricate balance between the proliferative DHT and the antiproliferative 3β-Adiol controls prostatic growth via the cognate receptors, it is important to define the intraprostatic enzymes involved in the synthesis and metabolism of the two steroids. The first enzyme shown to be involved in prereceptor regulation of steroid hormone action in the prostate was steroid 5α-reductase type 2 (18). This enzyme catalyzes the conversion of plasma-borne testosterone to the more potent androgen DHT, which is crucial for AR action from the time of fetal development throughout life (19).

Here, we found that 17βHSD6 uses DHT as substrate and that the product of the reaction is 3β-Adiol. In cell-based ERβ assays, coexpression of 17βHSD6 results in activation of ERβ by DHT as well as 3α-Adiol. The observed 3α-hydroxysteroid epimerase activity of 17βHSD6 with C19 and C21 3α-hydroxysteroids has

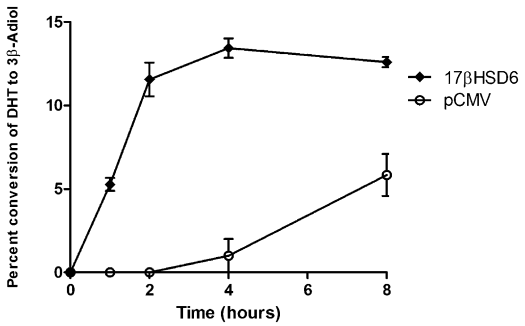

Fig. 3. DHT is converted to 3β-Adiol by 17βHSD6 in intact cells. Expression plasmids encoding 17βHSD6 or empty vector were transfected into HEK-293 cells on day 1. Conversion of DHT to 3β-Adiol was assessed on day 3 using tritiated DHT (20 nM) as substrate. Product quantitation was performed by TLC and radioactivity scanning. Data represent the mean ± SEM of three independent experiments.

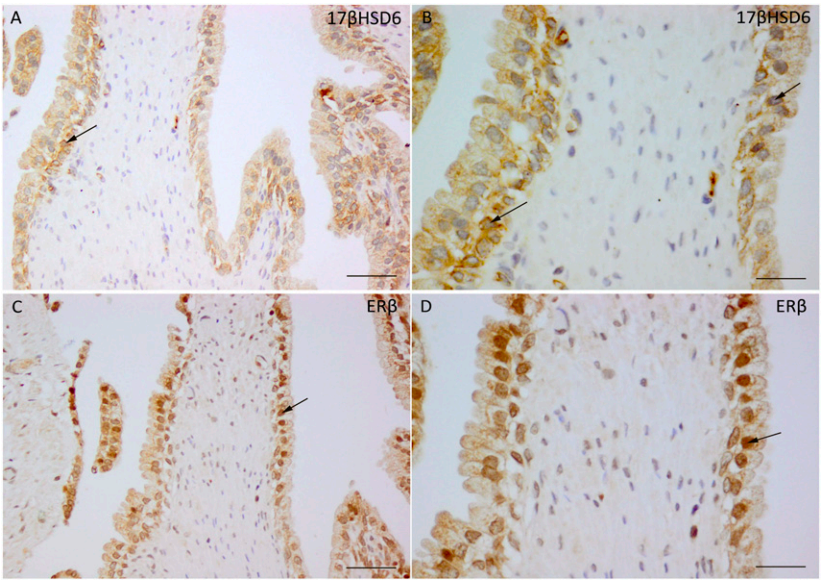

Fig. 4. Glandular epithelium of benign prostatic hyperplasia. (*A* and *B*) Positive perinuclear staining for 17βHSD6 (arrows). (*C* and *D*) Positive nuclear staining for ERβ (arrows). (Scale bars: *A* and *C*, 50 μm; *B* and *D*, 20 μm.)

previously been described using membrane preparations fortified with pyridine nucleotide cofactors or intact cells (10–12). Interestingly, prostatic 3α-Adiol levels are approximately five times lower than 3β-Adiol levels (3); hence, it is conceivable that this equilibrium favoring 3β-Adiol may be a result of the enzyme's epimerase activity. Another explanation could be that the 3α-Adiol formed by AKR1C2 is back converted to DHT by the enzyme's oxidative 3α-HSD activity. It has been shown that 3α-Adiol can be converted to DHT by 17βHSD6 and then activate AR (20). Thus, it is tempting to speculate that 17βHSD6 is involved in sustaining the intraprostatic balance of DHT and 3β-Adiol.

To add complexity to this interesting issue, it has been demonstrated that 3β-Adiol is inactivated in the prostate by CYP7B1, a 3β-hydroxysteroid-specific 6α/7α-hydroxylase (21). The enzyme is regulated in the epithelial cells of the ventral prostate during rat and mouse development (22); high levels of CYP7B1 (low 3β-Adiol) and no detectable ERβ are found in proliferating epithelial cells, whereas, in nonproliferating epithelial cells, CYP7B1 levels are low (high 3β-Adiol) and ERβ levels are high.

Immunohistochemical analysis of 17βHSD6 demonstrated perinuclear staining of the enzyme in the prostatic epithelial cells. This observation is in consonance with published data on the microsomal localization of 17βHSD6 following cellular fractionation by differential ultracentrifugation (13). This intracellular proximity of 17βHSD6 to ERβ may allow for efficient transfer of 3β-Adiol across the nuclear envelope to activate nuclear ERβ. The colocalization of 17βHSD6 with ERβ in the prostate and its ability to convert DHT to 3β-Adiol suggest that the enzyme serves a physiologically significant prereceptor regulatory role in prostate growth. This is further supported by the fact that 17βHSD6 and ERβ appear coordinately down-regulated in late-stage Gleason grade stage 4 and 5 prostate cancer. In this context, it is interesting to note the presence of potential ER/AP-1–binding sites within the 2-kb promoter region of the 17βHSD6 gene (23), suggesting that ERβ regulates the expression of the enzyme that produces its own ligand.

It would be of interest in the future to correlate the expression of 17βHSD6, ERβ, and CYP7B1 in diseased and normal prostate in an effort to further understand the role of these proteins in androgen- and estrogen-regulated growth of the gland and DHT and 3β-Adiol balance. In addition, it was recently reported that 5-androstene-3β,17β-diol functions as a selective ERβ modulator in microglia (24). This steroid is produced from dehydroepiandrosterone by 17βHSD14 whose expression is controlled by inhibitors and inducers of inflammatory responses. Hence, the potential role of 17βHSD14 and 5-androstene-3β,17β-diol in prostatic ERβ function remains to be investigated.

Materials and Methods

Human Samples. Prostate biopsies were obtained from the Department of Urology at Danderyd Hospital, Stockholm, Sweden. Biopsies were taken by Linda Waage, and the samples categorized for their Gleason grade (15) by Ulf Bergerheim. Samples were fixed in buffered paraformaldehyde, dehydrated, and imbedded in wax at Danderyd Hospital. We obtained the sections in a blind fashion. Samples from seven different patients with BPH (16), two patients with prostate cancer Gleason grade 3, three patients with prostate cancer Gleason grade 4, and two patients with prostate cancer Gleason grade 5 were used for this study.

Steroids. DHT (112 Ci/mmol) was purchased from Perkin-Elmer. E2, estradiol-17β; DHT, 17β-hydroxy-5α-androstan-3-one; 3α-Adiol, 5α-androstane-3α,17β-diol; and 3β-Adiol, 5α-androstane-3β,17β-diol were obtained from Steraloids.

ERβ Transactivation Assay. HEK-293 cells (ATCC CRL no. 1573) were maintained in DMEM with 10% FBS, 50 μg/mL kanamycin, and 10 mM Hepes, pH 7. On day

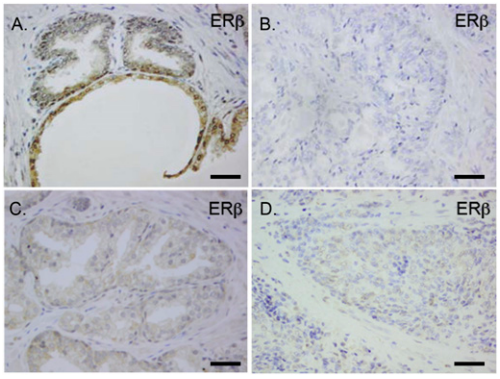

Fig. 5. ERβ in prostate cancer. (*A*) Gleason grade 3. (*B* and *C*) Gleason grade 4. (*D*) Gleason grade 5. The number of cells expressing ERβ is reduced in grade 3, and the expression of ERβ is lost in grades 4 and 5. (Scale bars, 50 μm.)

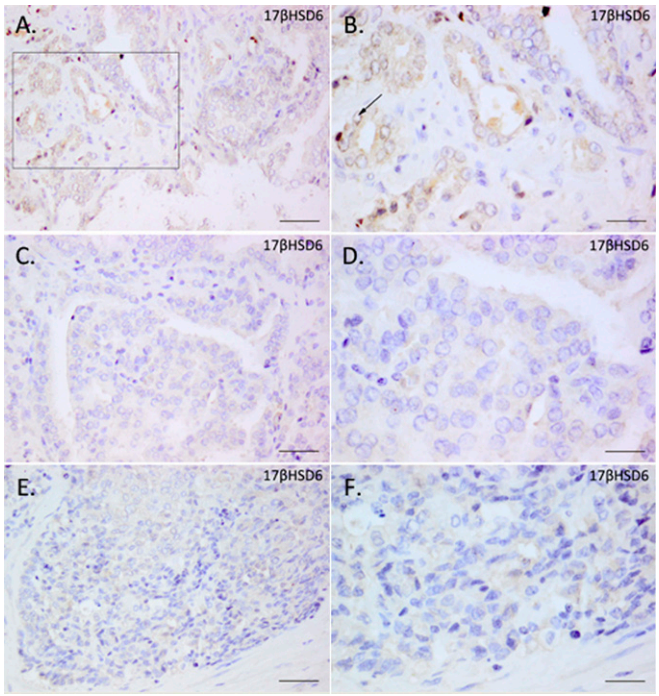

Fig. 6. 17βHSD6 in prostate cancer. (*A* and *B*) Gleason grade 3. The number of cells expressing the enzyme is decreased in the epithelium compared with BPH. (*C* and *D*) Gleason grade 4. (*E* and *F*) Gleason grade 5. No positive immunostaining is seen in the epithelial cells. (Scale bars: *A*, *C*, and *E*, 50 μm; *B*, *D*, and *F* 20 μm.)

0, HEK-293 cells were seeded in 24-well Costar plates containing 0.5 mL phenol red-free DMEM, 10% dextran-treated charcoal/dextran-treated FBS (HyClone), 50 μg/mL kanamycin, 2% glutamine, and 10 mM Hepes, pH 7. For incubation with 5α-reduced steroids, on day 1, the cells were transfected with pCMV–human ERβ1 (25) (40 ng/well), p3× (ERE)–TATA–luciferase (90 ng/well), pRSV–β-galactosidase (20 ng/well), and either pCMV–human 17βHSD6 (11) or pCMV vector (150 ng/well) using Fugene 6 (Roche) at a Fugene-to-DNA ratio of 4:1. Forty-eight hours after transfection, DHT, 3α-Adiol, 3β-Adiol, or E2 (1,000× stock solutions in DMSO) were added to final concentrations of 20 nM. After 16 additional hours of incubation, the cells were lysed and assayed for luciferase activity using a luciferase assay kit (Biovision) and β-galactosidase activity according to manufacturer's instructions using a PerkinElmer Victor ×4 plate reader (PerkinElmer). Relative level of transactivation was calculated by dividing luciferase units by β-galactosidase units. For dose–response curves with DHT or 3α-Adiol, cells were transfected as described above, and 48 h after transfection steroids were added to final concentrations of 1 nM to 10 μM. After an additional 8 h incubation, the cells were lysed and assayed for luciferase and β-galactosidase activities as described above. EC_{50} values were generated by fitting the data to a sigmoidal dose–response curve using the GraphPad Prism 6.0 software (GraphPad Software).

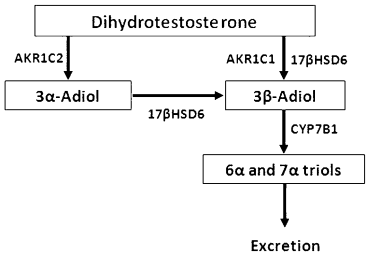

Fig. 7. Formation and inactivation of 3β-Adiol in human prostate.

Enzyme Assay for 17βHSD6 in Transfected HEK-293 Cells. HEK-293 cells were seeded in 24-well Costar plates on day 0 as described above. On day 1, cells were transfected with pCMV–17βHSD6 plasmid (270 ng/well) and pRSV–β-galactosidase (30 ng/well). Forty-eight hours after transfection, radioactive DHT was added to the individual wells (20 nM final concentration). One hundred microliters of medium was collected at different time points, and total lipids were extracted with 400 μL Folch reagent (chloroform/methanol: 2:1 vol/vol), vortexing and spinning at 14,000 × *g* for 5 min in a microcentrifuge. The organic phase was collected and evaporated in a speed-vac. The dried extract was redissolved in 40 μL Folch and spotted onto Partisil LK5D TLC plates (catalog no. 4855–821;Whatman). The plates were developed twice in chloroform–ethyl acetate (4:1 vol/vol) (8). Steroid metabolites were quantified using a BioScan AR-2000 Imaging System (Bioscan). After the final time point, cells were lysed and assayed for β-galactosidase activity to normalize for transfection efficiency.

Immunohistochemistry. For immunohistochemistry, two antibodies against 17βHSD6 were used: a rabbit polyclonal antibody (cat. no. ab62221; Abcam) raised against N-terminal amino acids 1–50 of human 17βHSD6 and a mouse polyclonal antibody (catalog no. H8630-B01P; Novus Biologicals) raised against the full-length human protein. For detection of ERβ, an antibody raised in chickens (ERβ 503 IgY) was used (26). Slides were deparaffinized with xylene and rehydrated through graded ethanol. For 17βHSD6, antigens were retrieved by boiling the slides in Tris·EDTA (Tris base 10 mM, EDTA 1 mM, 0.05% Tween 20, pH 9) buffer for 20 min in microwave oven. For ERβ, antigen was retrieved by heating at 97 °C for 15 min in citrate buffer (10 mM, pH 6) using a Pre Treatment module (ThermoScientific). After blocking endogenous peroxidase activity with 3% H_2O_2 in 50% methanol for 30 min, the slides were incubated in 3% BSA in PBS to block nonspecific binding followed by incubation with primary antibodies diluted in 3% BSA in PBS (17βHSD6, 1:300; ERβ, 1:200) overnight at room temperature. Slides were then washed with 0.1% Nonidet P-40 in PBS for 30 min and incubated with secondary antibodies. For ERβ, slides were incubated for 1 h with biotinylated goat anti-chicken antibody (Abcam) diluted to 1:200 in 3% BSA in PBS, washed in 0.1% Nonidet P-40 in PBS for 30 min, and incubated with Vectastain ABC (Vector Labs) for 1 h. For 17βHSD6, the slides were first incubated with Rabbit on Rodent HRP Polymer (Biocare) for 30 min followed by washing with 0.1% Nonidet P-40 in PBS for 30 min. After incubating with diaminobenzidine for 30 s and counterstaining with hematoxylin, the slides were dehydrated and mounted.

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